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Maturity and ripening-stage specific modulation of tomato (*Solanum lycopersicum*) fruit transcriptome

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Tomato (*Solanum lycopersicum*) fruit is a model to study molecular basis of fleshy fruit development and ripening. We profiled gene expression during fruit development (immature green and mature green fruit) and ripening (breaker stage onwards) program to obtain a global perspective of genes whose expression is modulated at each stage of fruit development and ripening. A custom made cDNA macroarray containing cDNAs representing various metabolic pathways, defense, signaling, transcription, transport, cell structure and cell wall related functions was developed and used to quantify changes in the abundance of different transcripts. About 34% of 1,066 unique expressed sequence tags (ESTs) printed on the macroarray were differentially expressed during tomato fruit ripening. Out of these, 25% genes classify under metabolism and protein biosynthesis/degradation related processes, while a significant proportion represented stress-responsive genes and about 44% represented genes with unknown functions. RNA gel blot analysis validated changes in a few representative genes. Although the mature green fruit was found transcriptionally quiescent, the K-means cluster analysis highlighted coordinated up or down regulation of genes during progressive ripening; emphasizing that ripening is a transcriptionally active process. Many stress-related genes were found upregulated, suggesting their role in the fruit ripening program.

Introduction

Fruit maturation and ripening are genetically regulated processes, which involve complex interplay of plant hormones and growth regulators with numerous biological and environmental factors.[1-7] Analysis of transgenic and mutant tomatoes has provided insight into the fundamental processes of plant fruit development and ripening and allowed genetic intervention for better fruit attributes.[2,8-13] The physiological and biochemical studies of a few decades have begun to provide the molecular basis of fruit development. Several transcriptional factors including MADS-box genes in addition to genes encoding ethylene biosynthesis and signaling components have been shown to impact fruit development and ripening.[14-16] These include *TAGL1*, *TAGL2*, *TAGL11* and *TAGL12* in fruit development,[17-19] *MADS-RIN* MADS box,[20,21] *Colorless nonripening* (*Cnr*) SPB box,[22] *HB-1* homeobox[23] and Aux/IAA transcriptional factor *IAA9*.[23]

Ripening of fleshy fruits involves evolution of ethylene, differentiation of chloroplasts into chromoplasts, accumulation of pigments such as carotene and lycopene, development of aroma and flavor, softening of fruit tissues and increased susceptibility to pathogens.[1,12] Studies aimed at averting the undesirable characteristics of ripening, namely, softening of fruit tissues and enhancing the desirable attributes of the fruit have resulted in a better understanding of the role of various genes, pathways and signaling molecules such as ethylene in fruit development.[1,11,12]

Tomato is a model for fleshy fruit development because of several desirable attributes: well-characterized tomato mutants that provide an amenable system for analyzing molecular aspects of fruit development;[2,4-6,12] small genome size of 960 Mbp, extensive mapping populations, physical and genetic maps, established transformation techniques and ease to propagate the plants both in vivo and in vitro;[25-27,29] availability of cDNA microarray along with companion databases for public deposition and retrieval of raw microarray data (http://bti.cornell.edu/). Thus, it was possible to carry out comparative studies to obtain insights into various fruit processes including tomato fruit development,[30-34] fruit set and early fruit tissue specialization,[35,36] transcriptional profiling of *high pigment-2dg* and *de-etiolated 1* tomato mutants[37,38] and response to *Calvibacter michiganesis*.[39] Microarrays have been used to study changes in fruit gene expression in tomato

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introgression[40] and comparative gene expression analysis in the Solanaceae family to which the tomato belongs.[41]

In the present study, a cDNA macroarray from a normalized cDNA library of ripening fruits of two processing tomato varieties was developed and used as a platform for analyzing global gene expression at six different stages encompassing fruit development and ripening in a processing variety of tomato (*Solanum lycopersicum* cv. Ohio 8245). We identify ESTs that show fruit stage-specific differential gene expression that was further validated by RNA gel blot analysis. Collectively, our results suggest that stress-associated genes along with ethylene signaling cascade genes are upregulated during the early stages of fruit ripening whereas the glycolytic pathway and chloroplast-related gene transcripts are enhanced during ripening. Late ripening cum senescence phase is associated with increases in the genes classified under ubiquitin-protease mediated protein degradation pathway.

Results

Development of tomato EST collection and macroarray. Inserts from over 1,600 cDNAs, which showed visually detectable hybridization signal with ^{32}P-labeled cDNA synthesized to total RNAs isolated from different stages of tomato fruit, were sequenced. The average length of the inserts was 500 bp. All sequences were submitted to the public EST databases (accession number CN550588–CN550664 and CD002010–CD003544) at the NCBI. Amongst these, a total of 1,536 ESTs were randomly selected for cDNA array preparation. These comprised 1401 ESTs (1,371 successfully sequenced tomato fruit cDNAs and 30 cDNAs for which sequencing failed) and the 135-control cDNA comprising the positive and negative standard spots.

For the 1,371 sequenced cDNAs, analysis for redundancy revealed 846 unique sequences while the remaining 525 Sequences were grouped in 202 clusters/220 contigs. Keeping in view the possible misassembles and single contigs generated in automated alignment of the sequences, a total of 1,066 (846 unique and 220 contigs) sequences were considered to be unique on the array. The presence of about 78% unique sequences indicated successful normalization with a larger number of distinct sequence representation in the library.

About 47% of 1,066 unique ESTs displayed similarities to genes/ESTs of known functionalities falling into 11 functional categories. The remaining 53% were assigned to unclassified, unknown or novel ESTs categories. ESTs having no definite predicted biological functions but showing homology to known sequences in databases (e-value of < 10^{-4}) were designated as 'unclassified' whereas those that showed similarity to sequences having no predicted function or showing low similarity matches to sequences in database were designated as 'unknown'. The ESTs that did not show homology to any of the sequences in the databases were designated as "novel" (**Table 1**).

Comparison of developmental stages of tomato using cDNA macroarrays. Crucial fruit development stages analyzed included maturation (immature green and mature green), ripening (BR, BR+3) and ripening/senescence (BR+7, BR+15) stages. Fully expanded leaf tissues were used as controls. Amongst the 616 unique, detectable ESTs selected for analysis of variance, 364 unique ESTs showed statistically significant differential expression at a FDR of 0.000427 (**Table 1**).[48] Examination of p-values for ESTs at Bonferroni cut-off ($p < 0.0000811$) resulted in the selection of 117 differentially expressed ESTs (**Table 2**). The differentially expressed ESTs represented members of all functional classes with the majority belonging to metabolism and protein biosynthesis/degradation related functions (12.1% and 11.8%, respectively). A large number of differentially expressed ESTs fell into unclassified (18.3%) and unknown (21.3%) categories (**Table 1**).

To decipher transcriptional activities associated with a given developmental stage, ESTs that showed at least a 2-fold change in the transcript levels were examined (**Fig. 1**). Higher percentage of ESTs showed increased transcript levels (8.2%) at immature green stage of the fruit with respect to the leaf tissue. Expectedly, a fruit specific protein (CD003222) showed a 4-fold increase in transcript levels whereas a photosystem II 10 kD polypeptide (CD003215) showed a two-fold decrease in transcript levels in the immature green fruit. ESTs showing at least two-fold increase in transcript levels at mature green stage (MG/IMG) were significantly low (2.4%) as compared to other stages; however, ESTs showing a two-fold decrease in transcript levels at this stage was high (17%). This decreased transcriptional activity at mature green stage suggested transcriptional quiescence of this stage with respect to the preceding, active cell division phase. Similar examination of other stages revealed that a significant number of ESTs (35%) that showed a two-fold decrease in transcript levels at the Br+3d (Br+3/Br) stage actually increased at Br+7 (Br+7/Br+3) stage. The low level of common ESTs, which showed similar transcript levels in various comparisons, indicated activity of different sets of genes in a stage specific manner.

Equal percentage of ESTs showing a two-fold increase or decrease (9.6%) in transcript levels at breaker stage indicated a state of active flux of transcription in fruits at this stage. As fruit ripening proceeded, more ESTs showed two-fold decrease in transcript levels (20% in Br+3d and 28% in Br+7d) with a lower number of ESTs showing two-fold increase (12% at Br+3d and 8% at Br+7d). Twenty three percent of the ESTs showing decrease in transcript levels at Br+7d showed increased transcript levels at Br+15d.

Developmentally regulated genes during fruit maturation and ripening. Analysis of expression profiles of the 364 differentially expressed ESTs in the fruit at various stages of development identified five clusters that appear to be regulated in a similar fashion (**Fig. 2**). Two of these clusters included ESTs that showed increased transcript levels at immature and mature green stages of fruit (cluster1 and cluster2), suggesting roles for these ESTs in the maturation phase of the fruit. The third cluster comprised of ESTs that showed higher transcript levels at Br stage and included ESTs that showed similarity to ripening related genes, such as 1-aminocyclopropane-1-carboxylate oxidase (ACO). The fourth and fifth clusters included ESTs showing increased transcript levels at Br+7 and Br+15 stages, respectively, suggesting a role for these ESTs in ripening as well as senescence related functions.

Table 1. Functional distribution of ESTs printed on macroarray and their expression in developing tomato fruit

Classification	ESTs		Unique detectable ESTs	Differentially expressed ESTs	
	Total	Unique		Total	% Unique ESTs
Cell structure and maintenance	16	16	5	2	0.6
Cell wall	29	21	12	9	2.5
Defense/stress response	78	61	46	30	8.4
DNA replication repair recombination	13	11	9	9	2.5
Energy	41	33	25	17	4.8
Ethylene response	37	11	7	3	0.8
Metabolism	155	117	72	43	12.1
Protein biosynthesis/degradation	106	88	66	42	11.8
Signal transduction	79	59	35	19	5.3
Transcription	55	42	20	13	3.7
Transport	52	44	24	9	2.5
Unclassified	306	240	121	65	18.3
Unknown	291	235	136	76	21.3
Novel	113	88	38	19	5.3
Nucleotide sequence unknown*	30	-	-	8	-
Total	1401	1066	616	364	100.0

Functional annotation for 1371 ESTs from a normalized ripening tomato fruit cDNA library were determined by comparing their sequences with the clusters of orthologous groups of proteins (COG), TIGR and MIPS databases (cut off e-value = 10^{-4}) and assigned to 14 categories with functions specified for 11 of these categories. Also shown are the number of unique ESTs among the total ESTs present on the array. An EST was considered detectable, if its signal intensity after hybridization was above the 95th percentile of the intensity of all blank spots. Number of ESTs showing differential expression were determined using ANOVA model as described in Materials and Methods. *Sequencing reactions failed. 135 spots were printed as control, of which 69 were negative control, 52 were positive with 14 as standards.

Fruit maturation associated genes. Cluster 1 and Cluster 2 (**Fig. 2A and B**) comprised of ESTs showing enhanced expression during the maturation phase (immature green and mature green phase). Cluster 1 (**Fig. 2A**) containing a set of 70 ESTs showed higher transcript levels at the immature green stage of the fruit (IMG/Leaf). This cluster (cluster 1) included 11 ESTs that are similar to genes having metabolism, 9 defense and stress related functions. Genes encoding for enzymes involved in primary metabolism such as amino acid biosynthesis included S-adenosylmethionine decarboxylase (CD003203); phytoene synthase (CD003472); trehalose-6-phosphate synthase (CD002955). Members of carbohydrate metabolism pathway such as enolase (CD003227), a putative ribokinase, a putative pyruvate kinase (CD002917) and an acetyl-CoA carboxylase (CD002728) were also differentially expressed.

Defense and stress related ESTs showing homology to genes encoding for heat shock protein (CD003245, CD003176), metallothionin type 2 (LeMTA, CD003172), a nonspecific lipid transfer protein (CD002278), a putative glutathione S transferase (CD002876) and an oxidative stress related gene (copper-zinc superoxide dismutase) were present in this group showing increased transcript levels at the early developmental stages.

The protein biosynthesis and degradation related 5 ESTs included homologs for ribosomal protein (CD003039), ubiquitin conjugating enzymes (CD002118), Ran binding protein 1 (CD002747) and a putative chaperonin (CD002558). Cell wall related 2 ESTs included an acid invertase (CD003202) and the energy related ESTs included homolog for lipoxygenase C (CD002724), ubiquinol-cytochrome C reductase complex (CD002476) and a vacuolar H^+-pumping ATPase (16 kD proteolipid, CD002473). An EST similar to the gene encoding for bZIP DNA binding protein (CD002855) and categorized as having transcription related function showed a 1.4-fold increase in transcript levels at the immature green stage. ESTs categorized as unclassified, unknown and novel included 11, 13 and 4 members, respectively, in this cluster.

Cluster 2 (**Fig. 2B**) was characterized by 82 ESTs with increased transcript levels at MG stage in comparison to immature green fruit stage. Transcripts of these ESTs remained largely unchanged during the ripening stages (Br, Br+3d, Br+7d) but increased during senescence (Br+15d). This expression profile was abounded by 14 ESTs (**Table 2**) implicated in protein biosynthesis and included homologs to ribosomal proteins, a SUI1 protein (CD002221), a translation initiation factor (eIF-5A 2, CD002375) and an ubiquitin-conjugating enzyme (CD002315).

The defense related 5 ESTs in cluster 2 included those homologous to genes encoding for a metallothionin-like protein (CD002101), a salt stress related SLT1 protein (CD002142), a pathogenesis-related NP24 protein precursor (CD003221) and a catalase isozyme 1 (CD002379). The metabolism-related 8 ESTs included a putative polyketide synthase (CD003488), epoxide hydrolase (CD002057), a putative chalcone isomerase (CD002862), nucleotide sugar epimerase like protein (CD003078) and an alcohol dehydrogenase class III homolog (CD002895). ESTs with cell wall-related function included pectinesterase1 precursor homolog (CD003231).

Table 2. Identity of 117 ESTs showing statistically significant differential expression at Bonferroni value ($p \leq 0.0000811$) during fruit maturation and ripening

Functional category	Cluster	Putative function	Fold change						p value
Accession number			I/L	M/I	B/M	+3/B	+7/+3	+15/+7	
Cell Wall									
CD003083	1	Putative protein	0.81	0.48	1.03	**2.20**	0.37	2.11	1.6926E-10
CD003231	2	Pectinesterase 1 precursor	1.92	**2.67**	0.54	0.99	0.52	1.41	3.5055E-08
CD003180	3	Polygalacturonase 2A precursor	0.79	0.74	1.72	**1.86**	0.49	1.05	1.0347E-06
CD002899	4	Putative protein	0.74	0.81	0.91	**1.24**	0.84	0.78	4.5681E-08
Defense/stress response									
CD003172	1	Metallothionein-like protein type 2	1.31	1.15	0.64	**1.79**	0.49	1.13	1.6067E-06
CD003176	1	Heat shock cognate 70 Kd protein 1	0.97	1.53	0.70	**1.89**	0.52	0.87	3.1658E-06
CD003229	1	Copper-zinc superoxide dismutase	1.44	0.67	0.98	**2.26**	0.55	1.10	7.9682E-06
CD003245	1	Hsp20.0 protein	1.48	0.72	2.22	**2.17**	0.30	1.24	3.6704E-08
CD002541	1	Dermal glycoprotein precursor	1.50	0.66	0.92	1.30	0.50	**1.54**	2.0399E-05
CD002876	1	Gene C-7 protein	0.82	1.19	0.61	**1.93**	0.52	1.16	2.5006E-05
CD002101	2	Metallothionein-like protein	0.83	1.03	0.68	1.24	0.41	**1.58**	5.2814E-10
CD002142	2	SLT1 protein	0.74	1.09	0.72	0.74	0.34	**2.47**	6.2883E-06
CD002379	2	Catalase isozyme 1	1.00	1.46	0.71	1.11	0.27	**1.60**	1.2172E-07
CD002740	3	Avr9/Cf-9 rapidly elicited protein	1.07	0.11	1.20	**3.25**	0.60	2.76	2.6118E-07
CD002883	3	hsr201 protein, hypersensitivity-related	0.65	0.31	**2.54**	1.69	0.75	1.59	2.7454E-06
CD002914	3	EST542130 tomato callus	**2.36**	0.45	2.19	0.84	0.36	1.39	3.1281E-06
CD002850	4	Putative Ozone-responsive stress-related protein	0.72	0.63	0.78	**1.87**	0.76	1.06	2.2076E-05
CD002941	4	Late-embryogenesis protein lea5	0.84	0.58	1.00	**1.43**	0.73	1.20	1.7418E-08
Energy									
CD002724	1	Lipoxygenase	0.96	0.64	0.85	**3.03**	0.48	1.35	1.5356E-07
CD003215	2	Photosystem II 10 kDa polypeptide	0.26	1.67	1.13	1.56	0.31	**1.68**	2.2333E-07
CD002834	3	Tubulin beta-2 chain	2.95	0.69	1.64	0.26	0.43	**2.26**	7.2793E-06
CD002910	3	RING-finger protein	5.19	0.63	2.60	0.49	0.15	**4.08**	1.1481E-07
CD002177	4	Expressed protein	0.93	0.55	0.43	1.23	**1.62**	1.07	2.5E-05
CD002496	5	Dihydrolipoamide dehydrogenase precursor	0.81	0.97	0.73	0.60	1.15	**1.29**	3.2468E-06
CD003254	5	Cytochrome b5	0.85	0.44	1.50	0.22	1.48	**3.00**	3.6019E-05
Metabolism									
CD002057	2	Epoxide hydrolase	1.44	0.98	0.51	0.94	0.22	**1.82**	2.1321E-06
CD002862	2	Similar to chalcone-flavonone isomerase	0.64	0.95	0.85	0.36	0.54	**1.30**	1.4776E-05
CD002841	2	Putative acyl-CoA synthetase	1.06	1.01	0.44	1.00	1.00	**1.72**	5.1006E-05
CD003078	2	Nucleotide sugar epimerase-like protein	1.43	0.91	0.62	1.12	0.21	**1.50**	1.6549E-05
CD002030	3	Selenocysteine methyltransferase	0.71	0.37	1.46	**1.65**	0.71	1.22	3.3149E-07
CD002078	3	Aspartic proteinase	0.77	0.35	2.13	0.69	0.69	**2.86**	1.701E-09
CD003246	3	Adenosylhomocysteinase	1.19	0.77	**2.42**	1.81	0.31	1.38	8.8558E-11
CD002872	3	Expressed protein	1.07	0.38	1.37	1.31	0.53	**1.65**	7.1973E-08
CD003084	3	Cystathionine-gamma-synthase	0.94	0.55	1.27	0.94	0.64	**1.67**	1.1497E-10

Bold represents the highest fold-change observed between consecutive stages during fruit development. I/L (IMG/Leaf); M/I (MG/IMG); B/M (Br/MG); +3/B (Br+3d/Br); +7/+3 (Br+7d/Br+3d); +15/+7 (Br+15d/Br+7d). See Table 1 Supplementary data for the complete list of differentially expressed genes during fruit development.

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CD002275	4	Acetolactate synthase II	0.70	**1.10**	0.68	1.09	0.87	0.84	2.7509E-05
CD002810	4	Putative protein	1.06	0.25	0.33	**2.38**	1.41	1.53	1.4954E-06
CD002842	4	Putative aldolase	0.72	0.79	0.62	**2.25**	0.69	1.02	6.7117E-06
CD002793	5	3-phosphoshikimate 1-carboxyvinyltransferase	0.64	1.61	1.65	0.46	0.91	**1.85**	2.1772E-06
CD003085	5	Expressed protein	0.62	0.92	0.88	0.34	**2.08**	0.46	9.8931E-08
Ethylene response									
CD003225	3	1-aminocyclopropane-1-carboxylate oxidase	1.01	1.62	**3.09**	1.49	0.40	0.98	2.964E-08
CD003249	3	Ripening protein E8	1.39	1.28	**3.84**	1.45	0.45	1.84	4.0883E-09
Signal transduction									
CD002963	3	MAP kinase 4 (MPK4)	0.91	0.39	1.43	**1.64**	0.70	1.04	1.6139E-08
CD002105	4	Putative protein	1.02	0.37	0.76	**1.61**	0.77	1.31	2.2299E-09
CD003467	5	ADP-Ribosylation factor 1	**2.18**	1.86	0.33	0.66	1.05	0.65	1.0317E-06
Protein biosynthesis/degradation									
CD002684	2	Ubiquitin precursor	0.83	0.79	0.77	1.16	0.56	**1.56**	2.7024E-08
CD002785	2	60S Ribosomal protein L30	1.41	1.38	0.86	1.00	1.00	**1.62**	1.5867E-06
CD002851	2	Hypothetical protein	0.93	0.70	0.56	1.07	0.17	**6.06**	1.125E-07
CD002521	3	30S Ribosomal protein S5	0.77	0.38	**1.52**	1.43	1.16	0.99	1.393E-06
CD002898	4	Ribosomal protein precursor-like	0.74	0.62	0.82	**1.61**	0.83	1.15	1.5858E-07
CD002890	4	60S Ribosomal protein L18A	0.64	0.66	0.68	**1.96**	0.80	1.28	3.3901E-05
CD003122	4	Cysteine proteinase	0.93	0.52	0.98	**1.52**	0.50	0.96	1.5359E-05
CD003437	5	Ribosomal protein S15	0.71	0.94	0.86	0.27	0.70	0.27	1.1018E-07
CD002864	5	Putative 60S Ribosomal protein L13A	0.85	1.06	0.67	0.18	**3.64**	1.07	8.8282E-06
CD003396		Ribosome-like protein	0.50	1.12	0.44	0.90	1.00	1	4.64E-06
CD003089	5	60S Ribosomal protein L30	0.93	0.71	0.67	0.54	**1.75**	0.35	4.0088E-05
Transcription									
CD002855	1	bZIP DNA-binding protein	1.42	0.69	0.59	**1.40**	0.65	0.99	7.67E-06
CD002126	2	Putative small nuclear ribonucleoprotein E	1.75	1.11	0.74	0.76	0.39	**1.53**	1.758E-05
CD003482	2	RRM-containing protein	0.98	1.63	0.80	**1.38**	0.21	3.38	3.144E-06
CD002877	2	Expressed protein	3.11	0.81	1.11	0.63	0.12	**1.83**	1.0583E-09
CD002980	4	RNA-binding protein	0.72	0.73	0.88	**1.39**	0.74	1.10	1.7495E-05
Transport									
CD003493	2	Importin alpha2	1.18	1.14	0.77	1.05	0.43	**1.44**	2.3566E-05
CD002346	5	Af10-protein	0.88	0.91	0.60	0.09	**16.6**	0.38	3.3207E-05
DNA replication repair recombination									
CD002318	2	DNA helicase-like	0.65	0.65	0.68	1.27	0.37	**2.88**	1.5575E-06
CD002875	3	Expressed protein	1.30	0.87	**1.55**	0.71	0.39	1.45	3.851E-07
CD002856	4	AtPH1-like protein	1.42	0.39	0.55	**2.02**	0.92	1.10	3.4276E-07
Novel									
CD003186	1	Novel	1.09	1.06	0.75	**1.58**	0.44	1.14	1.7836E-06
CD002381	2	Novel	1.35	1.02	0.42	0.81	0.18	**3.86**	4.5435E-05
CD002939	3	Novel	0.46	0.43	**1.73**	0.72	0.94	1.71	5.1018E-11
CD003541	5	Novel	0.72	0.76	**0.96**	0.42	0.88	**0.96**	5.8728E-06
Unclassified									

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CD002084	1	Putative protein	1.65	0.89	0.57	**1.75**	0.42	0.89	4.9369E-05
CD002483	1	Hypothetical protein	1.18	1.34	0.91	**1.81**	0.27	1.15	1.5526E-06
CD003184	2	Expressed protein	0.86	1.22	0.72	1.33	0.42	**1.98**	2.1212E-06
CD003192	2	Hypothetical protein	1.31	**1.78**	0.89	1.47	0.39	1.37	1.4714E-05
CD002281	2	Putative protein	0.79	0.80	0.61	0.82	0.95	**1.46**	2.8524E-05
CD002500	2	Translationally controlled tumor protein homolog	**1.90**	1.10	0.78	0.77	0.45	1.13	1.6582E-10
CD002776	2	Putative protein	1.00	1.00	0.88	0.25	1.00	**16.42**	2.1302E-06
CD002715	3	Putative protein	0.86	0.14	3.78	0.31	2.85	1.38	5.1583E-06
CD002317	4	P0677H08.7 [*Oryza sativa*]	0.68	0.61	0.89	**1.58**	0.67	0.91	1.9642E-05
CD003038	4	Putative protein	0.87	0.60	0.99	**2.50**	0.85	0.80	3.0688E-07
CD003047	4	Expressed protein	0.85	0.64	1.00	1.23	0.81	**1.31**	5.3887E-05
CD003120	4	Galactokinase like protein	0.71	0.67	0.90	**1.78**	0.87	1.03	1.0175E-07
CD002979	4	Putative protein	0.94	0.40	0.91	1.60	0.82	**1.76**	6.7433E-08
CD003373	5	Putative protein	1.24	1.23	0.44	0.06	1.37	**2.73**	3.0312E-06
CD002746	5	Putative protein	0.48	0.97	**4.56**	0.07	4.03	1.87	1.4565E-05
CD003033	5	Probable ATP-dependent permease	1.98	0.48	0.90	0.29	1.64	**2.18**	1.1702E-06
Unknown									
CD003500	1	Guanosine pentaphosphatase	1.16	0.60	0.56	**1.78**	0.60	1.37	6.6369E-08
CD002942	1	CG14770 gene	1.03	0.69	0.80	**1.31**	0.56	1.01	6.8153E-11
CD002952	5	Xylose isomerase	11.86	1.52	0.47	0.31	**1.86**	0.81	9.5871E-06
CD002861	5	Phosphatidate cytidylyltransferase	0.97	0.78	0.97	0.15	**1.50**	1.44	6.6696E-06
CD002901	4	Unnamed protein product	0.93	0.42	0.85	**2.31**	0.66	1.12	1.0041E-07
CD002902	4	RNA-binding protein	0.72	0.54	0.70	**2.36**	0.64	1.45	5.3824E-12
CD003068	3	Hypothetical ORF	0.51	0.56	**2.32**	1.62	0.48	2.20	5.9413E-07
CD002835	4	Elongation initiation factor 5C	1.06	0.36	0.92	**2.98**	0.62	0.59	2.0163E-06
CD002827	3	Unknown	1.32	0.28	**2.25**	1.04	0.65	1.00	1.5884E-05
CD002829	3	Erythema protein	**3.99**	0.53	2.04	0.48	0.35	1.79	1.1683E-07
CD002962	3	ABC transporter	1.00	0.44	**1.65**	1.04	0.69	1.45	2.5024E-07
CD003008	4	Competence-induced protein	0.64	0.49	1.06	**1.70**	0.98	1.05	2.9551E-07
CD002999	2	Ubiquitin specific protease 27	0.95	0.56	**1.33**	1.07	0.75	1.27	3.2826E-05
CD003049	5	Fibrillin-1	0.49	0.87	1.23	0.65	**1.23**	0.92	3.5453E-05
CD003087	4	CLE7	0.73	0.64	0.83	0.94	**1.34**	0.91	1.2939E-07
CD003090	4	Putative Heat shock transcription factor	0.73	1.20	0.13	**2.71**	1.35	0.07	2.1232E-05
CD002228	2	Unknown protein	**3.27**	1.01	0.61	0.65	0.46	3.02	1.60288E-05
CD002352	3	Unknown protein	3.51	0.69	**4.49**	0.77	1.69	0.39	4.18475E-05
CD002498	2	Similarity to RNA-binding protein	2.26	1.29	0.61	0.50	0.10	**4.72**	9.88761E-07
CD002717	3	Hypothetical protein	0.85	0.92	**2.66**	0.57	0.66	2.39	1.42422E-06
CD002745	1	OSJNBa0006B20.5	1.37	0.51	0.82	**2.17**	0.63	1.14	1.20546E-05
CD002811	4	Beta-galactosidase	0.89	0.72	0.66	**2.00**	0.64	1.04	2.21182E-05
CD002817	5	Trypsin precursor AiJ3	0.79	0.32	1.06	0.44	**3.54**	0.72	4.96366E-05
CD003020	3	Hypothetical gene	1.78	0.18	**1.89**	2.62	0.62	0.99	7.1264E-06
CD003037	1	Unknown protein	**1.95**	1.05	0.40	1.00	1.00	1.75	2.03586E-06
CD003208	1	Unknown protein	1.58	0.93	0.70	1.65	0.50	**1.66**	6.57474E-06

Bold represents the highest fold-change observed between consecutive stages during fruit development. I/L (IMG/Leaf); M/I (MG/IMG); B/M (Br/MG); +3/B (Br+3d/Br); +7/+3 (Br+7d/Br+3d); +15/+7 (Br+15d/Br+7d). See Table 1 Supplementary data for the complete list of differentially expressed genes during fruit development.

Table 2. Identity of 117 ESTs showing statistically significant differential expression at Bonferroni value ($p \leq 0.0000811$) during fruit maturation and ripening

CD003495	5	Probable cytochrome P450 monooxygenase	0.42	1.58	2.17	0.47	1.01	**2.25**	6.2696E-07
CD003080	4	Glycoprotein B	0.71	0.63	1.00	**1.63**	0.74	1.13	2.8417E-06
CD002986	1	OSJNBa0073L04.6	0.90	0.81	1.07	**1.35**	0.63	1.28	3.8666E-05
CD002982	4	Arginase	1.01	0.59	0.88	**1.20**	0.95	1.05	5.2927E-06
1F7	1	No sequence information	1.26	0.85	0.86	**1.39**	0.28	1.05	1.92138E-05

Bold represents the highest fold-change observed between consecutive stages during fruit development. I/L (IMG/Leaf); M/I (MG/IMG); B/M (Br/MG); +3/B (Br+3d/Br); +7/+3 (Br+7d/Br+3d); +15/+7 (Br+15d/Br+7d). See Table 1 Supplementary data for the complete list of differentially expressed genes during fruit development.

Transcription-related 6 ESTs in this cluster included homologs of a putative CCCH-type zinc finger protein (CD003350), putative small nuclear ribonucleoprotein E (CD002126), a putative RNA binding protein (CD003482) and ABI3-interacting protein 2 (CD002100). This category also included five transport-related ESTs homologs such as importin alpha2 (CD003493), a vacuolar ATP synthase subunit G_2 (CD003188) and an ADP ribosylation factor1 (CD002295). An ethylene responsive element binding protein (EREBP4, CD003062) was also upregulated at this stage. The categories of unclassified, unknown and novel ESTs in this cluster included 24, 7 and 5 ESTs, respectively.

Thus an induction of genes involved in various cellular processes during the early maturation stages of the fruit is evident. More ESTs (30 in number) showed an increase in transcript levels than a decrease (13 in number) at the immature green stage of the fruit in comparison to the leaf tissue. The mature green fruit appears to be transcriptionally quiescent because a large number of ESTs either did not show a significant change in the transcript level or decreased (**Fig. 1**).

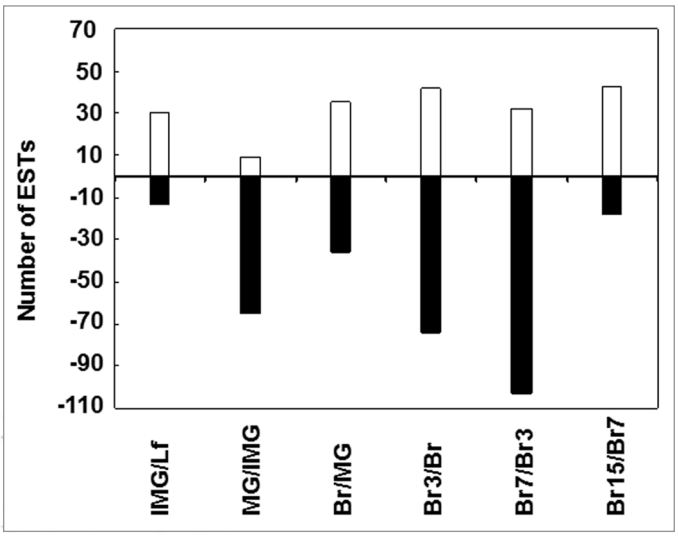

Figure 1. Transcriptional regulation during tomato fruit maturation and ripening. Expression ratios for each EST were calculated for successive development and ripening stages. Lf, IMG, MG, Br, Br3, Br7 and Br15 represent leaf, immature green, mature green, Br, Br+3d, Br+7d and Br+15d. Genes showing differential expression ($p \leq 0.05$) and more than two fold increase or decrease in transcript levels between indicated stages of fruit development are displayed. Open bar: Upregulated ESTs, Black bar: Downregulated ESTs.

Genes associated with onset of ripening. Breaker (Br) stage marks the beginning of the ripening process. A set of 66 ESTs showing an increase at the Br stage over the mature green stage grouped together in the cluster analysis (**Fig. 2C and cluster 3**). The transcript levels of these ESTs declined during the mature green stage of the fruit but increased in the following Br stage, remaining at a steady level during the ripening stage (Br+3d), decreasing at the later stages of ripening (Br+7d) and then showing an increase during the senescence stage (Br+15d).

Cluster 3 (**Fig. 2C**) included genes that are induced at the onset of fruit ripening including those involved in ethylene response (ripening protein E8 homolog, CD003249), cell wall metabolism (polygalacturonase PG2A precursor, CD003180) and a fruit specific protein (2A11) with unknown function (CD003222). The defense and stress related ESTs included an ethylene-induced and hypersensitive response hsr201 protein (CD002883) that is a homolog of Avr9/Cf-9 rapidly elicited protein (CD002740).

Metabolism-related ESTs (8 in number) in this cluster included amino acid biosynthesis genes such as selenocysteine methyltransferases (CD002030), adenosylhomocysteinase (CD003246), lysine decarboxylase like protein (CD002881) and cystathionine-γ-synthase (CD003084). Cell wall related ESTs such as actin depolymerizing factor (CD002777) and a tubulin-beta2 chain homolog (CD002834) showed 3.5-fold and 1.5 fold increases, respectively, at Br stage compared to the mature green stage.

The protein biosynthesis and degradation related 7 ESTs in this cluster showed homology to genes encoding for ribosomal proteins and a chloroplast elongation factor TuB (CD002957). An aspartic proteinase homolog (CD002078) also belongs to this category and increased by two-fold from MG to Br stage with an additional 3-fold increase registered at the Br+15d stage. The signal transduction related ESTs included homologs to a gene encoding for MAP kinase 4 (CD002963) and an auxin-responsive protein (CD002798). An EST similar to gene encoding for a RING zinc finger protein (CD002523) and a putative vesicle transport protein (CD002692) were representative for the transcription and transport related functional category in this cluster. ESTs categorized as unclassified, unknown or novel included 8, 18 and 1 EST, respectively, within this cluster.

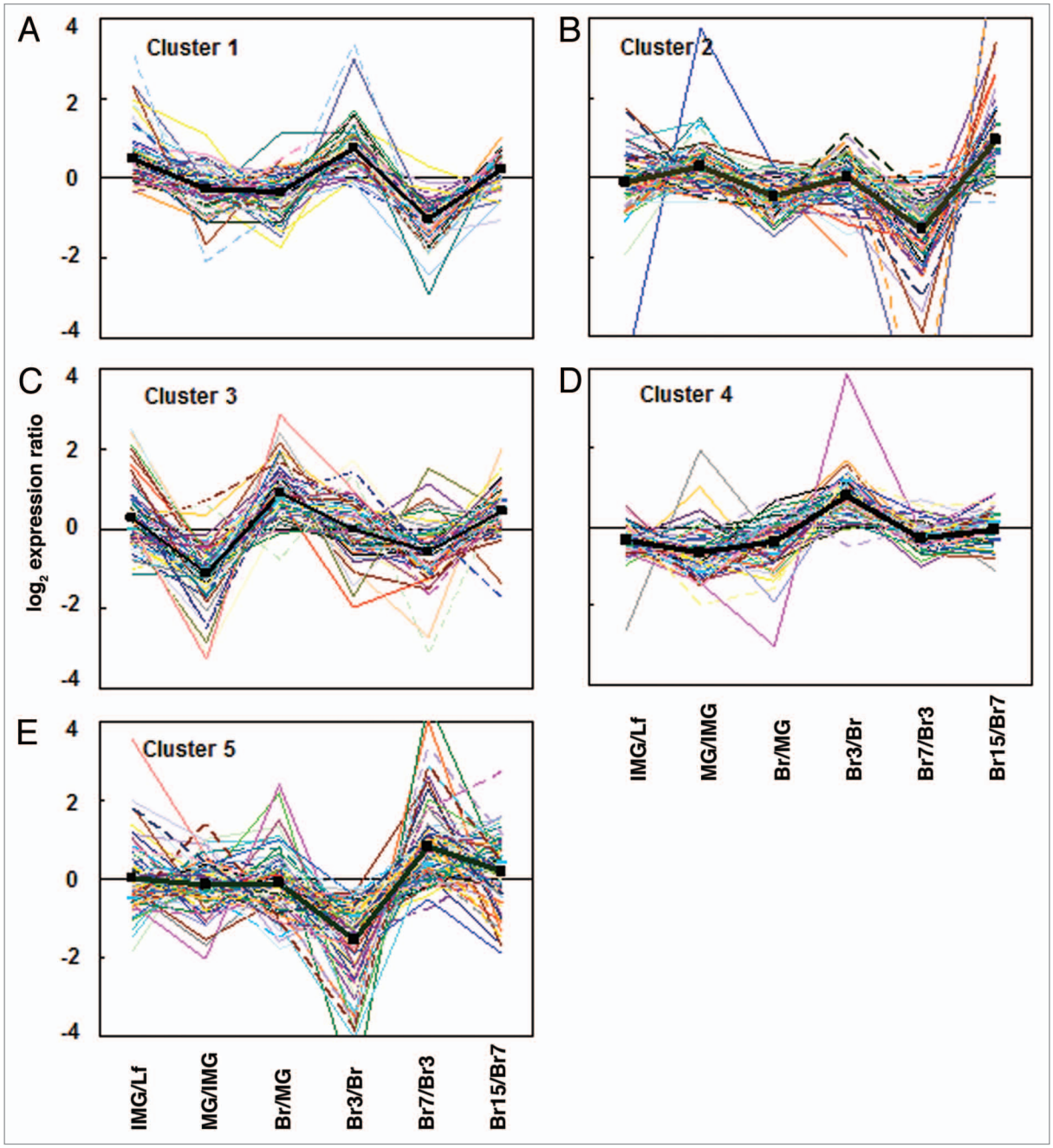

Figure 2. Expression profiling of differentially expressed ESTs during fruit maturation and ripening. Expression ratios for 364 differentially expressed ($p \leq 0.05$) ESTs at successive stages were calculated from normalized signal intensities for each EST and K-means clustering analysis performed using Gene Cluster v2.11 software.[59] The expression patterns for ESTs in each cluster are shown along with their average expression profiles (Thick line with filled squares). The stage keys are as described in legend to Figure 1. Clusters (A and B) represent fruit maturation-related genes, cluster (C) the onset of fruit ripening-related genes and cluster (D), and cluster (E) the ripening and senescence-related genes, respectively.

Genes associated with fruit ripening and senescence. The ESTs specific for fruit ripening and senescence exhibited two different expression profiles. In one of these two profiles, the transcript levels showed a pattern with an increase at the Br+3d stage over Br stage followed by a decline at the Br+7d and Br+15d stages. The second expression profile belonged to ESTs that included ESTs whose transcript level was higher at Br stage compared to Br+3d stage, followed by a further increase as the fruit entered the late ripening and senescence phase (Br+7d and Br+15d stages).

The ripening associated expression profile (**Fig. 2D and cluster 4**) included 67 ESTs. This profile was characterized by ESTs showing increased transcript levels at Br+3d stage with respect to the Br and Br+7d stage. The profile comprised of ESTs from different functional categories (**Table 2**). The metabolism related ESTs (6 in number) included an aldolase (CD002842), an

ascorbate peroxidase (CD002967) and an acetolactate synthase II homolog (CD002275). Defense related ESTs (5 in number) included homologs to STLS11 protein (CD003100), a wound-induced protein Sn-1 (CD003052), a probable wound-induced protein (CD002106), a stress-induced protein (CD002850) and a late-embryogenesis protein, Lea5 (CD002941).

Ribosomal proteins abounded the ESTs involved in protein biosynthesis and degradation category (9 in number). A chaperonin, CPN60 (CD003468), a cysteine proteinase (CD003122) and a putative proteasome regulatory subunit (CD002966) that participates in protein folding and degradation were present in the protein biosynthesis and degradation category. A general transcription factor II B (CD002882) and an RNA binding homolog (CD002980) were members of this expression profile showing higher transcript levels at Br+3d stage.

Twenty-one ESTs in cluster 4 were categorized under ESTs with unknown function and 8 were unclassified. An examination of ESTs for distinct increase or decrease in the transcript levels at Br+3d stage revealed forty-two ESTs that showed a 2-fold increase in transcript levels at Br+3d stages over the Br stage of the fruit and 74 ESTs showed a two-fold decrease in transcript levels at Br+3d stage of the fruit (**Fig. 1**).

The second set of ripening and senescence associated genes included 79 ESTs (**Fig. 2E and cluster 5**). The transcript levels of most of these ESTs were low at the IM, MG and Br stages, decreased at the Br+3d stage but increased at Br+7d and Br+15d stages. This cluster included ESTs (**Table 2**) showing similarity to defense and stress related (8 in number) genes such as metallothionin like proteins (LeMTA, CD003207), glutaredoxin (CD002909), a non-specific lipid transfer protein and lipid transfer protein 2 (CD003004, CD003492), cytochrome P450 (CD002672) and a pathogenesis related protein (CD002708). The metabolism related ESTs (10 in number) that showed increased transcript levels at Br+7d over Br+3d stage included phosphoshikimate 1-carboxyvinyltransferase (CD002793) and histidine decarboxylase (CD002766) homolog. ESTs with similarity to genes encoding for ribosomal proteins comprised the protein biosynthesis and degradation category (8 in number), along with ESTs showing similarity to an ubiquitin activating enzyme E1 (CD002227) and an ubiquitin conjugating enzyme E2. Amongst signal transduction related ESTs, those similar to phytochrome B2 (CD002887), a DNA binding protein 4 (CD002935), an auxin induced protein (CD002973) and a serine/threonine protein phosphatase (CD002421) showed increased transcript level at the Br stage with a further increase at the Br+7d stage. An EST with similarity to a cell wall related putative postsynaptic protein CRIPT (CD002569) also showed an increased transcript level at the Br and Br+7d stages. Amongst ESTs categorized as energy related, dihydrolipoamide dehydrogenase precursor (CD002496) and a cytochrome b5 (CD003254) showed increased transcript level at Br+7d stage. The unclassified, unknown and novel EST categories included 13, 15 and 8 ESTs, respectively.

Validation of macroarray data by RNA blots hybridization. Expression patterns of several ripening related genes were consistent with published data. However, to further confirm efficacy of array results we performed northern blot analysis (**Fig. 3**). Transcript levels of EST homologs to genes encoding for heat shock protein (CD003245), metallothionin like protein (CD002101), lipoxygenase C (LoxC; CD002724), a fruit specific protein (CD003222) and a beta fructofuranosidase EST were determined. A two-fold increase in transcript levels was observed for the heat shock protein (CD003245) at Br and Br+3d stages whereas the metallothionin (CD002101) showed a ~2.5 fold increase in the transcript levels at Br+3d stage. Lipoxygenase (LoxC) isoform showed a 2-fold increase at Br stage which remained high through the ripening stage and then decreased at the senescence stage. A fruit specific protein of unknown function (CD003222) showed an 8-fold increase in the immature green stage of the fruit in comparison to the leaf tissue but the increase in the fruit from young fruit to ripening stages was not significant. Invertase transcripts registered a 1.5-fold increase at Br+3d stage onwards till the senescence stages. These results validate the macroarray data described above.

Discussion

The development of cDNA microarray analysis[50] has been used to quantify transcriptome analyses of many organisms including *Arabidopsis thaliana*,[51] rice,[52] and other plants.[53,54] Several oligonucleotide based array (TOM2, Agilent-022270 Tomato Gene Expression Microarray, Affymetrix Tomato Genome Array) for tomato have been used for comparative gene expression patterns of tomato with other Solanaceae species including tomato mutants (high pigment-2dg; higher ascorbic acid content genotypes, parthenocarpic and anthocyanin producing fruits), transgenic lines transformed with either inositol polyphosphate 5-phosphatase or yeast ySAMdc and fruits treated with jasmonic acid (Geo Datasets, Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/sites/entrez). The availability of tomato genome sequences in the near future should greatly enhance the use of this technology to identify genes regulating tomato growth and development including fruit ripening.[29,55]

The microarray technology, however, requires specialized reagents and equipment that are not easily available in many laboratories throughout the world. We printed cDNAs representing over 1,000 unigenes obtained from a normalized cDNA library to develop a cDNA macroarray,[46] and tested its potential to quantify and compare gene expression changes during tomato fruit development and maturation. About 47% of the ESTs on the array belonged to 11 different functional categories and the remaining were either unclassified or unknown. About 8% ESTs that showed no sequence matches to sequences deposited in the public databases were considered novel genes. Profiling polyamine-mediated gene expression in transgenic tomato fruits, we have reported that polyamines function as anabolic growth regulators.[46,56-59]

Here we characterized changes in transcriptional patterns during tomato fruit maturation and ripening. Quantification of transcripts revealed that about 60% (364 out of 616) of unique ESTs were differentially regulated during maturation and ripening processes. Among the 364 differentially expressed genes, 117

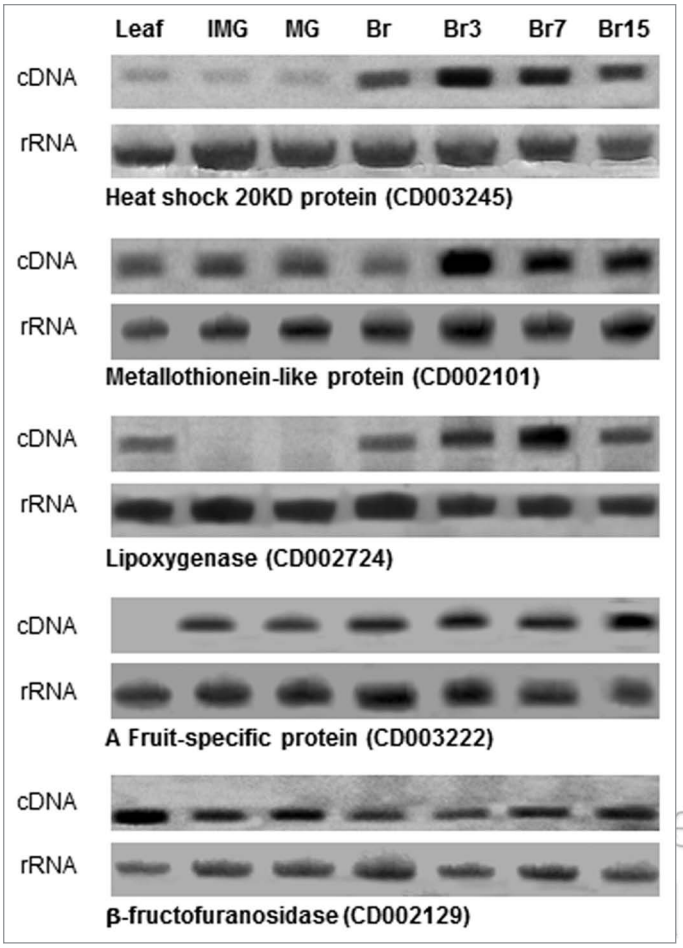

Figure 3. Verification of array expression patterns by northern analysis. Relative transcript abundance as determined by northern blots for the 5 ESTs identified as differentially expressed by macroarray in leaf, IMG, MG, Br, Br+3d, Br+7d and Br+15d are shown. Identities of ESTs are as follows: (A) CD003245, Heat shock 20 KD protein, (B) CD002101, Metallothionein-like protein type 2 LeMTB, (C) CD002724, Lipoxygenase (Lox C), chloroplast precursor, (D) CD003222, Fruit-specific protein and (E) CD002129, Minor allergen, beta-fructofuranosidase precursor.

were considered statistically significant ($p \leq 0.0000811$, pnorm ≥ 0.05) and the remaining 247 ($0.0000811 \leq p \leq 0.05$, pnorm ≥ 0.05) were classified as genes of interest.

Clustering of gene expression data generated in micro- and macro-array experiments is an effective method to obtain a holistic view of the temporal expression changes occurring within an organism due to genetic or epigenetic regulation.[60] This approach groups genes based on the similarity in their expression patterns. K-means clustering analysis of the 364 ESTs identified as differentially expressed or interesting were grouped into five different profiles to obtain an insight into the temporal changes occurring in a coordinated fashion over the six stages of fruit maturation and ripening studied here (**Fig. 2**). Each of these expression profiles was characterized by increased transcript levels at one or more stages of the fruit development and ripening, which allowed classifying them as early fruit maturation, on-set of fruit ripening and ripening and senescence associated ESTs.

The early fruit maturation specific category carried many ESTs that have been implicated in biotic and abiotic defense related functions. These included ESTs with similarity to genes encoding for metallothionins, heat shock cognate proteins and oxidative stress related proteins. Some of these ESTs were also found to show increased level at later stages of ripening and senescence. Metallothionins are cysteine rich polypeptides involved in metal detoxification and homeostasis in both prokaryotes and eukaryotes and induced by hormones, stress and heavy metals.[60] Differential expression of metallothionin-like proteins is known in apple,[62] banana,[63,64] grape,[65] strawberry,[66] citrus fruit[67] and kiwifruit.[68] These studies have indicated that different isoforms of the metallothionin genes may express in a stage specific manner during fruit ripening. The transcript levels of metallothionin type 2 genes have been shown to be high in the unripe green banana[63,64] whereas a metallothionin-like gene showed high expression in the early stages of fruit development in kiwifruit.[68] In tomato, we found higher transcript levels for metallothionin type 2 and metallothionein-like protein gene homologs in early developmental stage which increased further during ripening and senescence. Similarly, the differential expression of heat shock cognate proteins during fruit ripening has been previously documented.[44]

The increased transcript level of a chloroplast elongation factor at the onset of ripening in this study is interesting. The transition of chloroplast to chromoplast causes an increase in steady state levels of certain RNAs encoding for proteins destined for the chromoplasts,[70] thereby suggesting that this process is an active developmental program. Therefore, expression of the chloroplast elongation factor at the early ripening stages indicates an active translation process in this organelle.

Ripening of tomato fruit was also characterized by increased abundance of mitochondrial and chloroplast specific ribosomal mRNA. As the ripening fruit entered the senescence phase, the protein stabilization and degradation processes came into play. Thus, higher transcript levels for members of the ubiquitin-proteasome mediated protein degradation machinery along with programmed cell death-related cysteine proteinase became preponderant.[71]

Taken together, these results show that fruit ripening is a highly regulated developmental process and provide a window into the various cellular processes occurring at different stages of fruit maturation and ripening in a processing variety of tomato. Genes involved in diverse cellular processes such as primary and secondary metabolism, transcription, post-transcription modification, signaling pathway and translation, and those participating in physiological responses to biotic and abiotic stresses are expressed in a stage specific or transient manner.

We compared our data with the digital tomato expression database developed by the Cornell group (http://ted.bti.cornell.edu/). Since our library was made at the turning stage of the ripening fruit, we looked at the list of up and downregulated genes during fruit ripening and also the list of genes implicated in tomato fruit development, available at the TED site. Among the common ESTs present in both arrays, 18 downregulated and 33 upregulated ESTs during fruit ripening exhibited pattern similar

to that reported in the TED-database. However, a large proportion (53%) of the genes printed on the macroarray described here could not be assigned clear functionalities based on the sequences available in the public databases, along with 8% novel cDNAs, at the time of the preparation of this manuscript. These uncharacterized genes hold promise for providing additional tools to modulate process of fruit development and ripening.

Materials and Methods

Plant material. Tomato plants (*Solanum lycopersicum* cv. Ohio 8245, a processing variety) were grown in a green house as described previously.[42] This cultivar was a selection made in 1978 from F6 generation of a cross between Ohio 7870 and Heinz 722.[43] Fruits were harvested at immature green (25 days after pollination), mature green (when the fruit had acquired maximal size) and different ripening stages. For ripening stages, fruits were tagged at breaker (Br) stage and harvested after 0, 3, 7 and 15 days. Pericarps from three fruits of each stage were excised and frozen at -70°C. Fully expanded leaves were excised from the plant and frozen at -70°C.

Construction of cDNA library and sequence analysis. Fruits at turning stage from Heinz breeding lines 70,620 and 70,320 were used for total RNA extraction. The two lines differ in their fruit juice viscosity, Bostwick value of line 70,320 being 19.7 cm (thin viscosity) and line 70,620 12.2 cm (thick viscosity).[44] cDNA synthesis, equalization and cloning were a modification of Takahashi et al.[45] as described earlier.[46] Briefly, over 5,000 white transformed *E. coli* colonies were randomly picked and grown in 384 well plates containing LB broth and 50 μg/ml ampicillin. All colonies were printed in duplicate on nylon membranes using Biogrid II robotic workstation (Biorobotics, USA). Individual membranes were hybridized to ^{32}P-labeled cDNA synthesized from total RNA isolated from immature green, mature green, BR, turning ripe and over ripe tomato fruits to select clones that exhibited visually detectable signal. All bacterial colonies harboring various cDNA inserts that showed visually detectable signals were selected and characterized by DNA sequencing. Sequencing for cDNAs in excess of 1,600 was performed using ABI prism 3700 sequencer at the Purdue University DNA Sequencing Facility. Vector sequences were removed and the edited sequences were compared with those in the EST (dbEST), non-redundant nucleotide and protein databases (Genbank) at the National Center for Biotechnology Information (NCBI). The cut-off e-value of 10^{-4} was used. Stackpackv2.2 (Electric Genetics PTY Ltd.,) was used to analyze the consensus and unique sequences amongst the ESTs.

Construction of cDNA microarray and hybridization. A total of 1,536 cDNAs (1,401 from tomato fruit ESTs and 135 negative and positive controls) clones were PCR amplified as described.[46] All cDNAs were printed on PerForma II membranes (Genetix, USA) in duplicates using the 384-pin tool (0.4 mm pin diameter) in the Biogrid II robotic workstation in a 3 x 3 format (Biorobotics, USA). Macroarray hybridization experiments were performed in duplicates for all six stages of the fruit tissues and leaf tissues. Pericarps from three fruits of each stage were mixed and total RNA extracted. cDNA was reverse transcribed from total RNA (5 μg) using SuperscriptII reverse transcriptase (Invitrogen) with Oligo $(dT)_{12\text{-}18}$ (Gibco BRL, USA) as primer (0.5 μg) in a reaction mixture containing 1 mM each of dCTP, dTTP and dGTP along with 50 μCi of [α^{32}P] dATP following manufacturer's instructions. Unincorporated radioactive nucleotides were removed by filtration through G-50 Sephadex columns.[47] The cDNA arrays were washed with pre-hybridization buffer [0.25 M $NaHPO_4$ (pH 7.2), 5 mM EDTA, 4% SDS, 1x SSC] at 62°C for 2 h. Membranes were hybridized at 62°C for 22 h in solution containing 0.5 M $NaHPO_4$ (pH 7.2), 10 mM EDTA, 7% SDS and 1 x 10^6 to 4 x 10^6 cpm/ml of probe. Filters were washed twice for 10 min each [2x SSC and 0.1% SDS] and exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA USA) for 12 h.

Data acquisition and analysis. Data acquisition and analysis were done as described.[46] Dried filters were exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) for 12 h and scanned with the Typhoon Phosphorimager (Amersham Biosciences). Signal intensities were quantified using Imagene software (Biodiscovery) and corrected for background by subtracting the background mean intensity from the signal mean intensity. Data were normalized by dividing the signal intensity of each EST with median signal intensity value of the standards on each array. A cut-off threshold was set at 95th percentile of the blank signal intensities for signal detection. The effects of different stages on the ripening fruit were examined in an analysis of variance (ANOVA) modeling framework represented by $y_{ij} = \mu + \tau_i + \varepsilon_{ij}$, where y is the intensity of the EST as measured on the ith stage for the jth replicate after correction for the local background and normalization.[47] The parameter μ is the overall mean of the normalized values of that EST. Fixed effects for stage (τ_i) where i is the stage (IG, MG, BR, BR+3d, BR+7d and BR+15d) were examined. The error (ε_{ij}) represents random variation for the ith stage and the jth replicate (j = 1, … ,4). The null hypothesis assumed was that the expression levels were not different across different stages of development for a given EST. K-means cluster analysis was performed on the selected statistically significant ESTs using the Gene Cluster software package using expression ratios calculated from corrected signal intensities for the statistically significant ESTs ($p < 0.05$).

Northern analysis. Total RNA from leaf and fruit pericarp tissues was isolated and analyzed using the RNeasy Plant total RNA Kit (Qiagen Inc., Chatsworth, CA) as described.[44] Five micrograms of the total RNAs from each stage were separated on 1.2% agarose denaturing formaldehyde gels[45] and the separated RNA blotted on Hybond-N membrane (Amersham, UK). The blots were hybridized with α-^{32}P dCTP (3,000 Ci/mMol)-labeled probe at 42°C [50% formamide, 5x SSC, 50 mM sodium phosphate, pH 6.8, 0.1% sodium pyrophosphate, 0.1% SDS, 5x Denhardt's solution and 50 μg/ml Herring sperm DNA].[47] Radiolabeling of cDNA inserts from different ESTs was carried out using a random primer labeling kit (DECA Prime II, Ambion, Austin, TX), which were then purified on Sephadex G-50 columns (5.0 cm x 1.0 cm). After hybridization, membranes were washed twice for 15 min each [2x SSC, 0.1% SDS]

at 24°C and then twice for 10 min each [0.2x SSC, 0.1% SDS] at 62°C. Quantification of hybridization levels was performed using Typhoon 9600 scanner. The transcript levels were normalized to ribosomal RNA levels to account for loading differences.

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